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GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS ANTHRACIS RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL PROGRESS REPORT

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JULY 1989

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5212

University of Massachusetts
Amherst, Massachusetts 01003

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Massachusetts	6b. OFFICE SYMBOL *(If applicable)*
6c. ADDRESS *(City, State, and ZIP Code)* Amherst, MA 01003	7b. ADDRESS *(City, State, and ZIP Code)*
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research and Development Cmd	8b. OFFICE SYMBOL *(If applicable)*
8c. ADDRESS *(City, State, and ZIP Code)* Fort Detrick, Frederick, MD 21701-5012	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5212

10. SOURCE OF FUNDING NUMBERS			
PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62770A	3M16. 2770A871	AD	106

11. TITLE *(Include Security Classification)*
(U) Genetic and Physiological Studies of Bacillus anthracis Related to Development of an Improved Vaccine

12. PERSONAL AUTHOR(S)
Curtis B. Thorne

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT *(Year, Month, Day)*	15. PAGE COUNT
Annual Report	FROM 8/1/88 TO 7/31/89	1989 July	61

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	04	

18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*
Bacillus anthracis — RAI — Anthrax protective antigen
B. anthracis plasmids — Anthrax toxin
Bacillus conjugative plasmids — Transposon mutagenesis

19. ABSTRACT *(Continue on reverse if necessary and identify by block number)*

The primary objective of the research is to gain information and develop genetic systems that will contribute to development of an improved vaccine for anthrax. During the year represented by this report our research concentrated largely on (i) transposon mutagenesis in Bacillus anthracis with the transposition selection vector pTV1; (ii) further physical and genetic characterization of phage TP21, which is active on B. anthracis and whose prophage exists as a plasmid, and exploration of its potential as a vehicle for transposon mutagenesis; and (iii) further characterization of the conjugative plasmid, pLS20, of Bacillus subtilis (natto) and its ability to transfer plasmids among strains of B. anthracis, B. cereus, B. subtilis, and B. thuringiensis.

(continued)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. Miller	22b. TELEPHONE *(Include Area Code)* (301) 663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

To analyze the 184-kb B. anthracis toxin plasmid pXO1 genetically and physically, mutants were generated by transposing the MLS resistance transpson Tn917 to pXO1. Tn917 inserted more frequently into pXO1 than into the chromosome. Restriction analyses of several transposants showed that Tn917 inserted into the plasmid at a number of different sites. Deletions, probably mediated by Tn917, occurred in some of the pXO1::Tn917 derivatives. Other deletions were generated by transductional shortening using the generalized transducing phage CP-51.

The toxin plasmid carried by the Weybridge strain of B. anthracis differs from the plasmid in a variant of the strain designated Weybridge A. The phenotypes conferred upon host strains by the respective plasmids differed in several respects. To distinguish between the two we have designated the toxin plasmid in the original Weybridge strain as pXO1 and the one in Weybridge A as pXO1.1.

Several strains carrying pXO1.1::Tn917 derivatives and all tested strains carrying deletion derivatives of the tagged plasmid failed to produce the protective antigen component of the toxin. Also several strains were defective in producing edema factor and/or lethal factor. Restriction analyses of the transposants showed that Tn917 insertions into a 13.9-kb BamHI fragment (or an 18.1-kb PstI fragment) affected production of the toxin components. A few of the strains carrying the deletion derivatives were also aberrant in other phenotypes typically observed with strains carrying wild-type pXO1.1. These traits included the extent and rate of sporulation at 37°C and sensitivity to bacteriophage. We are optimistic that further experiments utilizing the tool of transposon mutagenesis will allow us to determine the location of genes responsible for the various known phenotypes and perhaps identify other genes on the plasmid which are currently cryptic.

Studies on phage TP21 whose prophage exists as a plasmid and whose host range includes many strains of B. anthracis, B. cereus, and B. thuringiensis have continued. This is the first demonstrated example of a Bacillus phage with a plasmid prophage. We have isolated a mutant of TP21 that is temperature sensitive for replication. The mutant has been tagged with Tn917 toward the goal of developing a convenient vector for transposon mutagenesis. Strains carrying the tagged temperature-sensitive mutant can be cured of the prophage by growth at the nonpermissive temperature, 42°C. Although theoretically this tagged phage mutant should be an ideal vector for transposon mutagenesis, we have had little success in transposition experiments. It is conceivable that the Tn917 carried by the phage mutant may be defective in transposition. We have recently isolated a second mutant of the phage that is temperature sensitive for replication and it has been tagged with Tn917. It will be tested for its effectiveness as a transposition selection vector in B. anthracis.

The Bacillus subtilis (natto) fertility plasmid pLS20 encodes functions required for conjugal transfer of plasmid DNA among a variety of Bacillus species including B. anthracis. We are continuing our studies with pLS20 in efforts to understand the mechanism of conjugal transfer among Bacillus cells. By means of transposon mutagenesis we have produced pLS20::Tn917 derivatives. They fall into three major groups with respect to their ability to mediate plasmid transfer. Class I plasmids are almost completely defective in mediating either self-transfer or transfer of other plasmids. Class II plasmids are unable to mediate self-transfer but are able to mediate the transfer of certain other plasmids. Class III plasmids are apparently unaffected in their ability to mediate conjugal plasmid transfer. The sites of Tn917 insertion in plasmids of the first two classes were found to be in a 10.8-kb BglII fragment. All transposon insertions which did not affect plasmid transfer functions were located outside this particular area. Results of cloning experiments have confirmed that the transfer genes are located within the 10.8-kb BglII fragment. The same region of pLS20 that is involved in transfer functions also appears to be involved in suppression of motility of host organisms. Cells harboring pLS20 were nonmotile, and electron photomicrographs revealed the absence of flagella. When cells were cured of the plasmid, motility was restored. Thus, a second function, i.e., suppression of motility, can be attributed to pLS20. Although B. anthracis cells do not have flagella and consequently are not motile, cells infected with pLS20 appeared under the electron microscope to have an altered cell surface.

SUMMARY

To analyze the 184-kb Bacillus anthracis toxin plasmid pXO1 genetically and physically, mutants were generated by transposing the MLS resistance transposon Tn917 to pXO1. As determined by DNA-DNA hybridizations, transposition of Tn917 to pXO1 was observed more frequently than transposition to the chromosome. Restriction analysis of several transposants showed that Tn917 inserted into a number of different sites. Three transposants exhibited deletions in pXO1::Tn917 which may be either spontaneous or Tn917-mediated. Deletions in some of the Tn917-tagged plasmids were also generated by transductional shortening using CP-51 phage. The sizes of the Tn917-tagged plasmids generated from these transductions ranged from 80 to 176 kb.

These studies have led to the discovery that the toxin plasmid present in the Weybridge strain of B. anthracis differs from that in a variant of the Weybridge strain designated Weybridge A. The phenotypes conferred upon the host by the respective plasmids differ with respect to rate and extent of sporulation at 37°C, sensitivity to bacteriophage, and growth characteristics on minimal medium. For that reason we have designated the toxin plasmid in the Weybridge strain as pXO1 and the one in the Weybridge A variant as pXO1.1.

One reason for genetically characterizing pXO1 is to locate regions on the plasmid which are responsible for several known phenotypes observed with B. anthracis. Several strains carrying pXO1.1::Tn917 and all the strains carrying deletion derivatives failed to produce protective antigen (PA). Restriction analysis of the transposants showed that Tn917 insertions into the 13.9-kb BamHI fragment (or 18.1-kb PstI fragment) affected PA production. Insertion into this region also appeared to affect production of the lethal factor and edema factor components of the anthrax toxin. A few of the strains containing the deletion derivatives were also aberrant in other phenotypes typically observed with strains carrying wild-type pXO1.1. These traits included the extent and rate of sporulation at 37°C and sensitivity to bacteriophage CP-51. Tn917 insertion into the 6.6-kb BamHI fragment (or a 9.3-kb PstI fragment) appeared to affect the host's sensitivity to CP-51. Of the six deletion derivatives examined, most were missing part or all of the following BamHI fragments: 34.8-kb, 13.9-kb, 7.4-kb, and 6.0-kb. As determined by homologous DNA-DNA hybridization, five of the six deletion derivatives contained deletions in the 34.8-kb BamHI fragment. We are optimistic that further ex-

periments utilizing the tool of transposon mutagenesis will allow us to determine with considerable accuracy the locations of genes responsible for the various known phenotypes. In addition we hope to be able to identify other genes on the toxin plasmid which are currently cryptic.

Bacillus thuringiensis subsp. kurstaki strain HD-1 carries a temperate phage whose prophage exists as a 46-kb plasmid. The phage, which has been designated TP21, appears to be the first demonstrated example in Bacillus species of a phage with a plasmid prophage. TP21 lysogens have been isolated which have Tn917 inserted in the prophage. Although insertion of Tn917 rendered some isolates defective, several isolates carrying this element produced viable phage which conferred erythromycin resistance upon lysogenized hosts.

Results of tests with TP21::Tn917 demonstrate a broad host range among B. anthracis, B. cereus and B. thuringiensis strains. TP21 lysogens were very stable during growth at high temperatures. A mutant of TP21, TP21c7, was isolated following NTG mutagenesis of a B. anthracis lysogen; this derivative appeared to be temperature sensitive for replication. B. cereus lysogens of TP21c7 grown at 30°C stably maintained the plasmid prophage. Strains cured of TP21c7 could be isolated from broth cultures of lysogens grown at 42°C. A derivative of TP21c7 was isolated which has Tn917 inserted. Lysogens of the transposon-tagged derivative, like those of the parental mutant phage, were stable at 30°C but could be cured of the phage at 42°C. Bacterial strains carrying TP21::Tn917 were resistant to MLS antibiotics. When cells lysogenized with wild-type TP21 were superinfected with TP21::Tn917, with selection for MLS resistance, the transposon-tagged prophage replaced the wild-type prophage. The resulting lysogens were subsequently cured of TP21c7::Tn917 prophage by growth at 42°C. Although theoretically TP21c7::Tn917 should be a useful vector for carrying out transposon mutagenesis in B. anthracis, as well as in B. cereus and B. thuringiensis, we have had little success in transposition experiments. It is conceivable that the Tn917 carried by TP21c7::Tn917 may be defective in transposition. We have recently isolated another mutant of TP21 that is temperature sensitive for replication and it has been tagged with Tn917. The new mutant will be tested for its effectiveness as a transposition selection vector in B. anthracis.

The Bacillus subtilis (natto) fertility plasmid pLS20 encodes functions required for conjugal transmission of plasmid DNA among a variety of Bacillus species including B. anthracis. We are continuing our studies of pLS20 in ef-

forts to understand the mechanism of conjugal transfer of plasmids among Bacillus cells. Utilization of the temperature-sensitive transposition selection vector pTV1 has allowed the isolation of a collection of pLS20::Tn917 derivatives. The majority of the Tn917 insertions lie within an approximately 15-kb region of pLS20, and tests with some of the insertion derivatives have revealed the necessity of this region of the plasmid for the transfer-positive phenotype of host cells. The pLS20::Tn917 derivatives have been divided into three major classes with respect to their ability to mediate plasmid transfer. Class I plasmids are almost completely defective in mediating either self-mobilization or mobilization of other plasmids. Class II plasmids are unable to mediate self-mobilization but are able to mediate the transfer of certain other plasmids. Class III plasmids are apparently unaffected with respect to their abilities to mediate conjugal DNA exchange. This group contains eight unique insertion derivatives. The first two classes of plasmid derivatives have the transposon inserted in a 10.8-kb BglII fragment. All transposon insertions which did not affect plasmid transfer functions were located outside this particular fragment. Results of cloning experiments have confirmed that the transfer genes are located within this 10.8-kb BglII fragment. Further experiments are underway to elucidate further the boundaries of the transfer region.

Until recently the only recognized phenotypic effects of pLS20 on host cells was the ability to mediate conjugal transfer of various plasmids. However, it now appears that the same region of pLS20 that is involved in transfer functions also appears to be involved in the suppression of motility of host organisms. Cells harboring pLS20 or the transposon-tagged derivatives appeared to be non-motile on soft agar plates and electron photomicrographs revealed the absence of flagella. When cells were cured of the plasmid or when they harbored certain deletion derivatives of pLS20::Tn917, motility was restored. Thus, apparently a second function, i.e., suppression of motility, can be attributed to pLS20. Whether this function is related to the conjugation process is not known. Although B. anthracis cells do not have flagella and consequently are not motile, cells infected with pLS20 appeared by electron microscopy to have an altered cell surface.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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ANNUAL PROGRESS REPORT

This is the fourth annual report submitted under contract DAMD17-85-C-5212. Research on the contract which began August 1, 1985 is a continuation of research previously carried out under contract DAMD17-80-C-0099.

During the year represented by this annual report our research concentrated largely on (i) transposon mutagenesis in B. anthracis with the transposition selection vector pTV1; (ii) further physical and genetic characterization of phage TP21, which is active on B. anthracis and whose prophage exists as a plasmid, and exploration of its potential as a vehicle for transposon mutagenesis; (iii) further characterization of the conjugative plasmid, pLS20, of Bacillus subtilis (natto) and its ability to transfer plasmids among B. anthracis, B. cereus, B. thuringiensis, and B. subtilis.

In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. Table 1 lists the bacterial strains, plasmids, and bacteriophages referred to in this report.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

$NBYCO_3$ agar: NBY agar with 7 g of $NaHCO_3$.

Phage assay (PA) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

LG broth: L broth with 1 g of glucose.

BHI broth: Brain heart infusion broth (Difco), 37 g.

Peptone diluent: Peptone (Difco), 10 g. Used for diluting phage and bacterial cells.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal 4NH: To Minimal 3 were added 40 mg of L-methionine and L-histidine, and 10 mg of nicotinamide.

Minimal XO: To Minimal 1 were added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

CA-agarose medium: CA-agarose medium for the detection of colonies producing protective antigen was prepared as follows: 0.75 g of agarose was added to 100 ml of CA broth (prepared as described by Thorne and Belton [11]) and the mixture was steamed until the agarose was dissolved. When the medium cooled to about 50° C, 1 ml of 20% glucose, 8 ml of 9% $NaHCO_3$, 6 ml of goat antiserum to B. anthracis, and 10 ml of horse serum were added. The medium was dispensed in petri plates (13 ml per plate) and the plates were left with their lids ajar while the agarose solidified. The plates were usable after 1 hr.

Antiserum. B. anthracis antiserum was kindly supplied by personnel of USAMRIID.

Propagation of bacteriophage. Bacteriophage CP-51ts45 for transduction was propagated by picking 5 plaques from an assay plate (B. cereus 569 indicator), suspending in 5 ml of 1% peptone and filtering through a Millipore HA membrane filter. Cells were grown in 25 ml of L broth containing 0.5% (wt/vol) glycerol at 30°C with slow shaking for 6 to 8 hours following a 10% transfer from an overnight culture in L broth with 15 µg of chloramphenicol per ml (to maintain the plasmid marker). One-half ml of cells and 0.5 ml of

phage suspension were added to 3 ml of soft NBY agar and layered over freshly poured plates of NBY agar containing 0.5% glycerol. Propagation plates were incubated at 30°C for at least 30 hours and the phage from each plate was harvested in 5 ml of PA broth. Cells were removed by centrifugation at 10,000 rpm for 15 min at 15°C, and the supernatant fluid was filtered through a Millipore HA membrane filter. Dimethyl sulfoxide was added to give a final concentration of 10% and $MgSO_4$ was added to give a final concentration of 0.02 M. Lysates were tested for sterility and stored at 15°C.

Cell-free lysates of TP21 were prepared by growing cultures of lysogens from a loop of cells or spores in 25 ml of BHI broth with 0.1% glycerol. Following 16-18 hours of incubation at 30°C the cultures were centrifuged to remove the cell debris and filtered through a 0.45-μm HA Millipore filter. Lysates of TP21c were prepared by lytic infection of B. cereus 569 in broth cultures. All lysates were confirmed to be free of bacterial contamination by plating samples on L agar.

Phage assays. For assaying bacteriophage CP-51ts45, 0.1 ml of B. cereus 569 spores (standard indicator) having 3×10^8 CFU/ml were added to 2.5 ml of soft PA agar. One-tenth ml of phage diluted in sterile 1% peptone was added to the mixture which was poured over PA agar plates. Assay plates were incubated at 30°C for 18 to 20 hours.

TP21 and TP21c were assayed by the soft agar overlay technique on NBY agar containing 0.1% glycerol with spores of B. cereus 569 as the indicator. Phage was diluted in 1% peptone and 0.1 ml was mixed with 0.1 ml of indicator in 2 ml of soft agar. Glycerol (0.1 % w/v) was also added to the top agar to improve the ability to detect plaques of phage TP21. Plates were incubated at 30°C and plaques were counted after 14 hours. Exposure of assay lawns to chloroform vapor made the plaques easier to see. This was done by adding chloroform to the lids of inverted assay plates (glass plates) and letting them stand at room temperature for 30 min. When the recently-discovered indicator, B. cereus 569 UM20-1C1, was used as indicator, the plaques were much more distinct and exposure of plates to chloroform vapor was not necessary.

Transduction of pTV1 with CP-51ts45. Recipient cells for transduction of pTV1 were grown in 25 ml of BHI broth containing 0.5% glycerol (w/v) with fast shaking at 37°C for 6 to 8 hours following a 10% transfer from an overnight culture. One-tenth ml of CP-51ts45 phage lysate and 0.1 ml of

recipient cells were spread together on HA Millipore filter membranes, and incubated on LE-agar (L-agar with 0.1 µg/ml of erythromycin) for 4 hours at 37°C to allow for phenotypic expression of plasmid encoded antibiotic resistance. The membranes were then transferred to L-agar plates containing selective levels of erythromycin and lincomycin (1 µg of erythromycin and 25 µg of lincomycin per ml) and incubation was continued at 37°C for 30 hours.

Chromosomal transduction with CP-51ts45. Recipient cells for transduction of chromosomal markers were grown in 25 ml of L broth containing 0.5% glycerol (w/v) at 37°C with fast shaking for 6 to 8 hours following a 10% transfer from an overnight culture. One-half ml of CP-51ts45 phage lysate and 0.5 ml of recipient cells were mixed together in a 20-mm cotton-plugged tube and incubated at 37°C for 30 minutes with fast shaking. For transducing the recipient to MLS^r, 0.1 ml of the transduction mixture was spread on an HA filter membrane placed on L agar supplemented with inducing concentrations of erythromycin (0.1 µg/ml). The plate was incubated for 4 hours at 37°C to allow phenotypic expression of antibiotic resistance. The membrane was then transferred to L agar containing 1 µg of erythromycin and 25 µg of lincomycin per ml to select for Tn917. For transducing auxotrophic mutants 0.1 ml of the transduction mixture was spread directly on appropriate minimal medium to select transductants inheriting the wild-type allele. The plates were scored after 2 days of incubation at 37°C.

Nitrosoguanidine mutagenesis. Bacteriophage TP21 was mutagenized by growing lysogens in medium containing N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at a final concentration of 100 µg/ml. A stock solution (1 mg/ml) was prepared by first dissolving 10 mg of NTG in 1 ml of acetone and then adding H_2O to 10 ml final volume. For use, 2.5 ml of this stock was added to 25 ml of BHI broth containing 0.1% glycerol.

Preparation of lysogens. Cell-free lysates of TP21 were assayed on lawns of the strains of which lysogens were desired. The lysogens were obtained from the centers of turbid plaques by stabbing plaques with sterile toothpicks and then streaking for isolation on PA agar plates.

Lysogens of the TP21 mutants TP21c-3, TP21c-7, TP21c-8 and TP21c-12 were prepared by streaking an L agar plate with growth from a lawn of cells in soft agar which had undiluted phage lysate spotted onto it. Colonies of presumptive lysogens were picked to both a master plate and to a soft agar lawn of *B. cereus* 569 spores. Those colonies which made a zone of clearing on

the B. cereus lawns were then tested for the presence of the prophage plasmid by agarose gel electrophoresis.

Detection of plasmid DNA. The procedures for extracting plasmid DNA and for electrophoresis were the same as those described in the last Annual Progress Report (July 31, 1988).

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions recommended by the supplier of the enzymes. Usually 10 to 20 µl of DNA (1.0 to 1.5 µg) in TES (pH 8.0) was added to 5 to 10 units of enzyme in a 1.5 ml Eppendorf tube. Appropriate amounts of distilled water and 10X buffer were added to give a total volume of 100 µl. Reaction mixtures were incubated in a 37°C water bath for 2 to 15 h. Digests were heated at 65°C for 10 minutes to stop reactions and then resolved on agarose gels. Molecular weights of DNA fragments were determined by comparing their mobilities to those of a kilobase ladder consisting of fragments ranging in size from 0.2 to 12.2 kb or a set of high molecular weight markers ranging in size from 8.3 to 48.5 kb. Both sets of DNA size standards were obtained from Bethesda Research Laboratories.

Procedures used in mating experiments:

(1) Matings in broth: Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient.

For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10 µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

(2) Matings on membranes: Donor and recipient cells were grown in 250-ml flasks containing 25 ml of BHI broth and incubated at 30°C on a reciprocal shaker, 80 excursions per min. Transfers (5%, v/v) from 14- to 16-hour cultures were grown for 5 hours. One ml of donor cells and 1 ml of recipient cells were mixed and 0.1-0.2-ml samples were spread onto Millipore DA or HA membranes (Millipore Corp., Bedford, MA) which were placed on nonselective medium for 5 hr. BHI agar was usually used if the recipients were B. anthracis, B. cereus, or B. thuringiensis. PA agar was usually used for B. subtilis, and LG agar was used when the matings involved B. subtilis natto. To determine the number of donor and recipient cells per membrane, the mixture was diluted in peptone and plated on the appropriate selective media. Control mixtures contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Plates were incubated at 30°C for 5 hours to allow mating and phenotypic expression. Membranes were subsequently transferred to agar plates containing tetracycline (for pBC16 transfer) and either rifampicin or streptomycin to select for recipients which had acquired the antibiotic resistance plasmid from the donor. To select for transfer of Tn917-containing plasmids, membranes were transferred to agar containing erythromycin and lincomycin, and either rifampicin or streptomycin. Colonies were scored after 1 to 2 days of incubation and transcipients were purified on the selective medium. The use of auxotrophically-marked strains facilitated unambiguous identification of transcipients. Frequency is expressed as the number of transcipients per donor.

Screening colonies for protective antigen production. Colonies were picked to plates of CA-agarose medium and incubated at 37°C in 20% CO_2 for

about 16 hours. A zone of precipitate formed around colonies that produced the protective antigen component of anthrax toxin (12).

Protective antigen (PA) assay. B. anthracis cells were grown overnight at 37°C with shaking in casamino acids (CA) broth containing 0.2% glucose, 0.27 mg $FeSO_4 \cdot 7H_2O$, selective levels of antibiotics (if the strains being tested carried Tn917-tagged plasmids), and an additional 40 µg/ml uracil (if B. anthracis Weybridge A UM23 derivatives were used). One-tenth ml was transferred to 100 ml of fresh CA broth supplemented as above and including 0.2% Norit A, and 0.72% $NaHCO_3$. The cultures were grown statically at 37°C for 27 hrs. Ten-ml samples of each culture were mixed with 0.5 ml of horse serum and filtered through Millipore HA membrane without a prefilter. The culture filtrates were diluted by serial two-fold dilutions and tested for PA antigen by an Ouchterlony double diffusion assay using goat antiserum prepared against B. anthracis (supplied by USAMRIID). The plates were incubated at 37°C for 24 hrs and 48 hrs with high humidity. The highest dilution which produced a visible precipitin line was taken as the end point.

Halo assay. To screen for protection antigen production, colonies were picked to CA-agarose immunoassay plates containing antiserum. The plates were incubated at 37°C with 20% CO_2 for 10 to 14 hrs. PA synthesis was indicated by the presence of a halo around the colony. The CA-agarose immunoassay plates were made by adding 0.75 g of Seakem GTG agarose (FMC Bioproducts, Rockland, ME) to 100 ml of CA broth. The solution was steamed until the agarose was dissolved and the solution was cooled to 47°C. The following reagents were subsequently added: 1 ml of 20% glucose, 0.1 ml of $FeSO_4 \cdot 7H_2O$ (2.7 mg/ml), 8 ml of 9% $NaHCO_3$, 10 ml of horse serum (Gibco Laboratory, Life Technology, Inc., Chagrin Falls, OH), 6 ml of goat antiserum to B. anthracis (supplied by USAMRIID), and an additional 40 µg of uracil per ml if B. anthracis Weybridge A UM23 derivatives were used.

Electroelution of restriction fragments from agarose gels. Following electrophoresis of the restriction endonuclease digests, the sections of the agarose gel containing DNA fragments to be eluted were cut out and then soaked in Tris-borate buffer (0.089 M Tris-hydroxide, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3) for 5 to 10 min. Electroelution of DNA was carried out using an Elutrap electro-separation chamber according to the manufacturer's instructions (Schleicher and Schuell, Inc., Keene, NH).

RESULTS AND DISCUSSION

I. Transposon mutagenesis in B. anthracis with the transposition selection vector pTV1

pTV1 is a transposition selection vector developed by Youngman (12). This 12.4-kb plasmid contains the Streptococcus faecalis transposon Tn917, which carries an erythromycin-inducible gene for MLS resistance. The plasmid also carries a chloramphenicol resistance determinant and is temperature sensitive for replication. It has been used to carry out transposon mutagenesis in B. subtilis and other Bacillus species into which it can be introduced by transformation or transduction. The fact that we have been able to transform B. cereus and B. anthracis by the protoplast transformation method as well as by electroporation opened up the possibility of using pTV1 for transposon mutagenesis in B. anthracis. At the time the last Annual Progress Report was prepared (July 1988) we had just initiated our work on transposon mutagenesis. That work has continued throughout the past year and the results are presented here.

Chromosomal mutagenesis

Introduction of pTV1 into plasmid-free derivatives of B. anthracis and B. cereus provided suitable host strains to determine the ability of Tn917 to insert within the chromosome and thus to utilize transposon mutagenesis as a tool that would facilitate genetic studies of B. anthracis. These studies have included the following aspects:

1. Induction and selection of Tn917 chromosomal insertions in liquid batch culture.

2. Recovery and phenotypic characterization of auxotrophic mutants resulting from Tn917 chromosomal insertions.

3. Confirmation of insertional mutagenesis by transduction with phage CP-51.

4. Test for chromosome transfer by conjugation using as potential donors strains with insertions of Tn917 in the chromosome and carrying Tn917-tagged pXO12.

Induction and selection of chromosomal insertions. Approximately 5 x 10^8 spores of strains into which pTV1 had been introduced by electroporation or by transduction, e.g., Weybridge A UM23-2(pXO1)$^-$ or B. cereus 569 UM20-1C1, were inoculated into 25 ml of BHI-0.5% glycerol broth containing 15 µg of chloramphenicol (Cm) and 0.1 µg of erythromycin (Em) per ml (the latter to induce transposition) in a 250-ml cotton-plugged flask which was incubated at 30°C on a shaker (130 rpm). After 16 to 18 hours 1.0 ml was transferred to 25 ml of BHI-glycerol broth containing selective levels of Em (1 µg/ml) and lincomycin (Lm, 25 µg/ml) and no chloramphenicol. The transferred culture was incubated in a 43°C water bath with shaking (160 rpm) for 4 hours. The culture was transferred in a similar manner three additional times. Following the fourth transfer the culture was incubated for 12 to 16 hours, and then 1 ml was transferred to 25 ml of L broth (no antibiotics) and incubated on the shaker at 30°C until sporulation occurred as observed by phase microscopy (3 to 4 days). This last step was omitted when an asporogenous mutant was used.

Once sporulation had occurred, the culture was centrifuged at 10,000 rpm for 10 min at 4°C (Sorvall SS-34 rotor) and the spores were resuspended in 5 ml of sterile water. The resuspended spores were heatshocked (65°C, 30 min) and 0.1-ml samples of appropriate dilutions were spread on plates of L-agar containing 0.1 µg of Em per ml. The plates were incubated at 30°C for 24 hours.

Cells in which transposition of Tn917 and loss of pTV1 had occurred were selected by replica plating the colonies to L agar containing selective concentrations of erythromycin and lincomycin and to L agar containing 15 µg of chloramphenicol per ml. The Em^r Lm^r Cm^s colonies were screened for auxotrophic mutants on minimal XO agar, and mutant phenotypes were determined by supplementing minimal XO agar with appropriate nutrients.

When spores were prepared following the mutagenesis procedure as described above, generally 95% or more were found to be transposants, i.e., Em^r Lm^r and Cm^s. Approximately 1% of the transposants tested were found to be auxotrophic mutants (one was a pigmented mutant). A description of the characterizable mutants isolated is given in Table 2.

To determine whether the mutants resulted from Tn917 insertions or whether they resulted from coincidental spontaneous mutations, transductions with CP-51 were performed as follows:

(i) CP-51 was propagated on each of the mutants and used to transduce Weybridge A UM23 Ura$^-$ or B. cereus 569 UM20-1, respectively, with selection for Em^r Lm^r transductants which were then tested for acquisition of the appropriate mutation. In each instance all of the MLS^r transductants tested (from 25 to 312 MLS^r colonies each) displayed the new mutant phenotype that had been acquired by the original transposant.

(ii) CP-51 was propagated on Weybridge A UM23 Ura$^-$ or B. cereus 569 UM20-1 and the respective auxotrophic mutants were transduced with selection for the wild-type allele. These were then tested for sensitivity to Em and Lm. In each instance all of the transductants tested (from 25 to 224) were sensitive to the antibiotics. These experiments show that transpositions of Tn917 from pTV1 to the chromosome do occur in B. anthracis and B. cereus and that pTV1 can be used as a tool to obtain insertion mutations.

Tests for transfer of chromosomal genes by conjugation. We have shown (3) that Tn917 can mediate cointegrate formation between the B. thuringiensis fertility plasmid pXO12 and the B. anthracis capsule plasmid pXO2 when both plasmids in the donor strain carry Tn917. Since transfer of large plasmids in the B. thuringiensis mating system apparently occurs via cointegrate intermediates, it seems possible that chromosomal transfer might occur in the same manner.

The availability of mutants carrying Tn917 insertions in the chromosome opened the possibility of determining whether transfer of chromosomal genes can occur by conjugation. To this end we constructed B. anthracis and B. cereus strains which contained pXO12::Tn917 and pBC16 and which carried Tn917-generated auxotrophic mutations. These strains were used as donors in matings with a variety of recipients which were auxotrophic as a result of point mutations. Selection was for transcipients which had inherited the wild-type allele of the mutated gene in the recipient. We also selected Tc^r transcipients as a measure of conjugal activity in the mating mixtures.

Further details of these experiments are not included here because only negative results were obtained. Although normal numbers of Tc^r transcipients were obtained from the mating mixtures we were not able to isolate any transcipients that had lost their original auxotrophic mutation. These experiments do not prove conclusively that chromosomal transfer does not occur by conjugation; they only provide evidence that the genes in question were not transferred. If we knew more about the locations of specific markers on the

chromosomes of B. anthracis and B. cereus, more conclusive tests for chromosomal transfer by conjugation could be carried out.

Transposon mutagenesis of pXO1

Several phenotypes have been attributed to the presence of the 184-kb toxin plasmid, pXO1, in B. anthracis. Weybridge A strains cured of pXO1 exhibit changed phenotypes with respect to extent and rate of sporulation, sensitivity to bacteriophage, toxin production, growth on minimal medium, and colony morphology (10). One of our reasons for studying the genetics of pXO1 is to identify the regions of the plasmid responsible for conferring these phenotypic characteristics upon the host strain. Another reason for studying pXO1 is to identify other phenotypes which may be associated with the presence of pXO1 in B. anthracis. Following are descriptions of advances made during the past year in the physical and genetic analysis of pXO1. Most of these studies involved the use of Tn917-tagged plasmids.

Classification of pXO1 from different B. anthracis strains. Weybridge UM44 exhibited different plasmid-derived phenotypic characteristics from those observed in Weybridge A strains. They differed in rate and extent of sporulation at 37°C, phage sensitivity, and growth characteristics on minimal medium. However, UM44-derived strains into which deleted pXO1::Tn917 derivatives from A UM23 were introduced showed characteristics similar to those typical of A UM23. No apparent differences were observed in BamHI restriction patterns of pXO1 from Weybridge UM44 and pXO1 from Weybridge A UM23 and UM14. However, there was a slight difference in the PstI and EcoRI restriction patterns of pXO1 from the two strains. Two new fragments were observed in PstI digests and in EcoRI digests of pXO1 from Weybridge UM44 compared to those of pXO1 from Weybridge A UM23. Because of differences observed in the toxin plasmid from different B. anthracis strains, we have suggested that the plasmid of Weybridge UM44 be designated pXO1 and the one from Weybridge A strains be designated pXO1.1. Similarly, the toxin plasmid from other B. anthracis strains will be designated pXO1.2, pXO1.3, etc., as they are characterized.

Size determination of pXO1.1. The size of pXO1.1 was determined by summation of restriction fragments generated by PstI and also by BamHI. The sizes of the restriction fragments were estimated by comparing their

mobilities in agarose gels to those of standard fragments of known size. Table 3 lists the number of fragments obtained from each restriction enzyme digestion and the estimated size of each fragment. The size of pXO1.1 estimated from BamHI digestions was 186.8 kb and the size estimated from PstI digestion was 181.5 kb. The average value was 184.1 kb. This value is in agreement with the average value of 184.5 kb obtained by D. Robertson using SstI and BamHI restriction enzymes (International Workshop on Anthrax, Winchester, England).

Generation of Tn917 insertions into pXO1.1. Transposition of Tn917 into pXO1.1 from pTV1 was carried out as described above. The presence of Tn917 on the plasmid was confirmed by DNA-DNA hybridizations using ^{32}P-labelled Tn917 as the probe. Approximately 75% of the MLS-resistant, chloramphenicol-sensitive colonies isolated from the transposon mutagenesis experiments carried Tn917 on pXO1.1. From these experiments 29 Weybridge A UM23 strains containing pXO1.1::Tn917 derivatives were isolated of which at least 13 occurred by independent transposition events.

Table 4 lists the B. anthracis transposants along with their phenotypic characteristics. A majority of these strains carrying Tn917-tagged pXO1.1 derivatives phenotypically resembled the wild-type pXO1.1-containing strain. Seven of these derivatives failed to produce protective antigen. CP-51 phage generated plaques on tp28, although the plaques were more turbid than the plaques produced on the (pXO1.1)$^-$ strain. This observation suggested that the phage was not as lytic in tp28 as in the (pXO1.1)$^-$ strain. The appearance of mutants altered with respect to protective antigen and CP-51 plaque formation indicated that the transposon inserted in or near the structural genes responsible for these characteristics or in regulatory regions.

Restriction analysis of Tn917-tagged pXO1.1 derivatives. Results from PstI and BamHI digestions of pXO1.1::Tn917 DNA from various A UM23 transposants are shown in Table 5. Analysis of these derivatives showed that Tn917 inserted into a number of different sites on pXO1.1. The new fragments generated from the PstI and BamHI digestions of the Tn917-tagged pXO1.1 derivatives contained Tn917 as confirmed by DNA-DNA hybridizations using ^{32}P-labelled pTV1 (extracted from B. subtilis PSL1 UM2) as the probe.

In several instances, e.g., tp1A, tp2A, tp15, tp27, and tp36, insertion of Tn917 did not affect any known plasmid-associated phenotypes. However, phenotypic alterations occurred in other transposants. Strains containing

tp21, tp29, tp32, and tp39 did not produce protective antigen. In addition, three of these PA$^-$ mutants, tp29, tp32, and tp39, were determined by Dr. S. Leppla of USAMRIID to be LF$^-$ or LF$^{+/-}$ and tp29 and tp39 were also found by him to be EF$^-$. All four of the PA$^-$ mutants contained an altered 13.9-kb BamHI fragment. Tn917 also inserted into the 13.9-kb BamHI fragment of pXO1.1 in tp27; however that strain synthesized PA. Preliminary data from EcoRI digestion of pXO1.1::Tn917 obtained from tp27 and tp32 showed Tn917 had inserted into different regions, thus perhaps, accounting for the differences in the observed phenotypes. These results suggested that the 13.9-kb BamHI fragment may contain a regulatory site for toxin production. As shown in Table 5, the tagged plasmids from tp21 and tp39 are missing more than one BamHI and PstI fragment. The PA$^-$ phenotype of these transposants may be caused by an alteration or deletion of one or more fragments.

The region of pXO1.1 responsible for susceptibility to CP-51 plaque formation may be located on the 6.6-kb BamHI fragment. The altered plasmid carried by Weybridge A UM23 tp28, which is apparently changed only in this phenotype, has Tn917 inserted in the 6.6-kb fragment.

Three of the transposants examined thus far, Weybridge A UM23 tp15, tp21, and tp39, exhibited deletions which may either be spontaneous or mediated by Tn917 insertion. The deletions ranged from 7 to 30 kb and appeared to be stable. When these transposants were grown over a period of time with and without erythromycin, no further deletions were observed. This phenomenon of Tn917-induced deletions has been observed in our laboratory with pLS20 containing Tn917 insertions.

Generation of B. anthracis strains containing deleted pXO1.1::Tn917 derivatives. B. anthracis strains containing deletion derivatives of pXO1::Tn917 were generated by CP-51-mediated transduction. Based on the size of its genome, CP-51 should package about 90 kb of DNA. CP-51 was propagated on various transposants and the lysates were used to transduce B. anthracis strains cured of pXO1 or pXO1.1. MLS-resistant transductants were isolated and the plasmid content was determined. In this experiment we isolated 37 strains carrying deletion derivatives of which at least 17 represented independent transductions.

All the B. anthracis strains carrying deleted pXO1.1::Tn917 derivatives were examined for phenotypic alterations. Table 6 lists these strains and their phenotypic characteristics. None of the strains harboring deletion

derivatives produced protective antigen. Five of the strains containing deletion derivatives were susceptible to plaque formation when infected with CP-51 and showed enhanced sporulation, phenotypes that are commonly observed with Weybridge A strains cured of pXO1.1. Two of these strains exhibited aberrant plaque morphologies (A UM23C1 tds2 and tds6). Two of the strains carrying deleted pXO1.1::Tn917 only exhibited enhanced sporulation. These results may be useful in further isolating regions of pXO1.1 responsible for plasmid-derived phenotypes.

Restriction analysis of Tn917-tagged pXO1.1 deletion derivatives. Comparisons of BamHI and PstI restriction patterns of the deletion derivatives with wild-type pXO1.1 or Tn917-tagged pXO1.1 derivatives from which the deletions were derived confirmed that deletions had occurred. The sizes of the deleted pXO1.1::Tn917 plasmids ranged from 80 to 176 kb (Table 7). The sizes of the deletion derivatives suggested that during transduction CP-51 may be packaging more than the size of its genome (ca. 90 kb). An analogous phenomenon was observed by Hendrickson and Duggan (5) in P1 transduction of F14 genes into F^- strains of E. coli .

The deleted fragments and the new fragments observed upon digestion of the deletion derivatives with BamHI and PstI are listed in Tables 8 and 9. The fragments which contained Tn917 were confirmed by DNA-DNA hybridization using ^{32}P-labelled pTV1 as the probe. From the BamHI restriction patterns of many of the deletants, the 34.8-kb fragment appeared to contain deletions. To confirm this, the deletants were probed with the 34.8-kb fragment. The probe was obtained by digesting pXO1.1 with BamHI, eluting the 34.8-kb fragment from agarose gels, and radiolabelling the fragment with ^{32}P-dGTP.

The deletion derivatives, tds1, tds2, and tds6, were derived from Weybridge A UM23 tp2A in which Tn917 was inserted into the 34.8-kb BamHI fragment (or more specifically into the 5.2-kb PstI fragment). Besides containing deletions, these derivatives contained new BamHI fragments the size of 18.7 kb, 20.7 kb and 17.3 kb, respectively. These fragments contained Tn917 and they were homologous to the 34.8-kb BamHI fragment. PstI digestion of these three shortened plasmids generated new fragments (5.8 kb, 15.2 kb, and 6.0 kb, respectively) which contained Tn917. The pXO1.1 deletion derivatives from tds4d and tds9 were derived from Weybridge A UM23 tp1A in which Tn917 was inserted into the 38.7-kb BamHI fragment, forming a 46.3-kb fragment. The 46.3-kb BamHI fragment from these two deletion derivatives also

contained Tn917 as determined by DNA-DNA hybridization. These same two deletion derivatives also contained newly generated BamHI fragments (28.7 kb and 27.8 kb, respectively) which were homologous with the 34.8-kb BamHI fragment from the parent plasmid, pXO1.1. The deletion derivative tds5 was missing the same BamHI fragments as the Tn917-tagged plasmid from which it was derived (contained in A UM23 tp21). Like tp21, tds5 produced a 44.2-kb BamHI fragment which contained Tn917 and was homologous to the 34.8-kb BamHI fragment. The difference between the two fragments is 9.4 kb which could be accounted for by the size of Tn917 (5.2 kb) and part of another fragment which may have lost its BamHI site.

Most of the deletion derivatives appeared to be missing part or all of the BamHI fragments located in the region of pXO1 thought to be associated with toxin production (34.8-kb, 6.0-kb, 13.8-kb, and 7.4-kb), thus rendering the strains PA^-. Evidence that the 6.6-kb BamHI fragment plays a role in the cell's sensitivity to CP-51 (as suggested by A UM23 tp28) is unclear. Weybridge A UM23C1 tds1, which is missing the 6.6-kb BamHI fragment, did not exhibit plaque formation when infected with CP-51. However tds2, tds4d, tds6, and tds9 all exhibited plaque formation, yet they appeared to contain the 6.6-kb fragment. This may imply that the sensitivity to CP-51 observed with the A UM23 tp28 strain is chromosomal-related, or that the Tn917-tagged pXO1.1 in tp28 contains a point mutation, or that there may be a second site on pXO1.1 which has an effect on sensitivity to the bacteriophage. Further analysis of the transposants and the deletants and more precise mapping of the different regions may help to elucidate these findings.

According to D. Robertson's map of pXO1, the origin of replication is contained in the 4.0-kb and the 7.1-kb BamHI fragments. These regions appear to be deleted from the 82-kb Tn917-tagged pXO1.1 deletion derivative from A UM23C1 tds1; however, the plasmid was stably maintained. Further analysis will be necessary to confirm these findings.

Growth requirements of Weybridge A UM23C1 tds5. The ability of this strain to synthesize PA could not be determined because it did not grow well in CA broth when incubated statically. To determine whether this was due to a new nutritional requirement or to an inability to grow under semi-anaerobic conditions, the strain was tested for the ability to grow under various conditions. Although cells grew well in CA broth contained in cotton-plugged shaken flasks, they grew poorly in CA broth, with or without sodium

bicarbonate, contained in cotton-plugged flasks incubated statically. In contrast, the strain grew well in L broth contained in cotton-plugged flasks incubated statically. Further analysis is needed to confirm these results. If these observations are reproducible, presumably a mutation has occurred and it will be important to determine whether the mutation is chromosomal or plasmid-borne.

II. Further studies with phage TP21 as a potential vector for transposon mutagenesis

We have continued our studies with phage TP21 whose prophage exists as a plasmid. This is the first reported example of such a phage for gram-positive bacteria. We first found TP21 in a strain of Bacillus thuringiensis subsp. kurstaki. The extrachromosomal nature of TP21 prophage makes TP21 valuable as a potential vector, especially since its broad host range includes B. anthracis. However, there appears to be considerable variation in the degree of sensitivity of various B. anthracis strains to the phage, with most strains being fairly insensitive. Following is a review of some of our studies with TP21 and an assessment of its potential for use as a vector, e.g., for transposon mutagenesis.

To determine which strains lie within the host range of TP21, two different strategies were employed. One procedure tested for lysis of cells in a spot test and the other procedure explored the ability of Tn917-tagged TP21 to infect cells and convert them to MLS resistance.

TP21c1 spot tests. In this procedure to determine the host range, a drop of lysate containing the clear-plaque mutant TP21c1 propagated on B. cereus 569 was spotted on a lawn of either cells or spores of each of the strains to be tested. After the lawns were incubated for a suitable period of time, any degree of turbidity less than that in the surrounding lawn of cells indicated that the strain may lie within the host range. A drop of B. cereus 569 cell-free culture filtrate was also spotted onto the test lawn to control for a response to phage or bateriocins carried by the host on which TP21c1 was propagated.

This procedure may not be sufficient to recognize all of the strains that fall within the host range. Strains which are lysogens of TP21 or of some related homoimmune phage would not be detected. Strains with very low

efficiencies of plating may not show any visible lysis. The results of this test, shown in Table 10, indicate that TP21 has a broad host range.

Ability of TP21::Tn917 to convert cells to MLS resistance. The second procedure employed to test the host range of TP21 made use of the Tn917-tagged derivative of the phage. TP21::Tn917 was mixed with cells of the strain to be tested and samples were plated on selective medium to select MLS-resistant cells. Obviously cells that were converted to MLS resistance were sensitive to the phage, i.e., the phage could adsorb to the cells and transfer DNA. This technique should have the capacity to detect strains in the host range of TP21, even strains that contain a homoimmune phage or give a very low efficiency of plating. The results of this test are included in Table 10.

The sensitivity of the test strain can be judged approximately by examining the number of erythromycin-resistant transductants produced by a TP21::Tn917 lysate. However, the number of MLS-resistant transductants can be influenced by other factors not related directly to the phage, such as restriction and modification systems.

Twelve strains of B. anthracis, six strains of B. cereus, and twenty strains of B. thuringiensis were used in these tests. The B. anthracis strains contained various genetic backgrounds with regard to their virulence factors. Examples of pXO1- and pXO2-containing strains, i.e., toxin producing or capsule producing strains, as well as cured derivatives were included. Of these eleven B. anthracis strains examined only one, ΔAmes-1, was not sensitive to TP21 as determined by this test or by the spot tests. The degree of sensitivity was extremely low for all strains of B. anthracis except the Pasteur strain 4229, which appeared to be about as sensitive as B. cereus 569. Most strains of B. cereus and B. thuringiensis were within the host range of TP21 as indicated by the results of these tests; however, there were considerable variations in sensitivity among the various strains.

Examination of the factors influencing sensitivity to TP21. Several factors can influence the sensitivity of a bacterial strain to phage. These include: (i) the availability of receptors on the cell wall, (ii) immunity of lysogenic bacteria to superinfection by homologous phages, and (iii) destruction of foreign DNA by restriction endonucleases . To try to understand which of these processes might be involved in the low sensitivity of most B. anthracis strains, we prepared lysates of the tagged phage from several strains of B. anthracis lysogenic for TP21::Tn917 and tested them for

the ability to transduce MLS resistance. Cell-free lysates of the transposon-tagged phage prepared from lysogenic cells of B. anthracis strains that exhibited low sensitivity to the phage were able to transduce MLS resistance to B. cereus 569 and in some instances to the homologous B. anthracis strain. The frequency of transduction for B. anthracis strains appeared unchanged or perhaps lower than the frequency of transduction obtained during the host-range tests.

This test ruled out restriction and modification as the reason for the low sensitivity of B. anthracis strains in general. It also ruled out the possibility that the MLS-resistant lysogens of these B. anthracis strains resulted from infection by rare host-range mutants of TP21::Tn917 which could recognize and adsorb to receptor sites different from those found in highly sensitive hosts. If the MLS-resistant isolates did carry a host-range mutant then the frequency of transduction of the homologous recipients should have increased over the values obtained originally.

Results of the above tests suggest the possibility that most cells in populations of the B. anthracis strains that appear to have a low sensitivity to TP21 are insensitive to the phage, and exposure of populations to transposon-tagged TP21 allows the selection of cell mutants that are sensitive to the phage. I believe we are in a position to test this possibility. As shown below, we were able to cure TP21 lysogens of B. cereus 569 and B. anthracis 4229 by first replacing the wild-type prophage with the prophage of a temperature-sensitive phage mutant that is tagged with Tn917 and then curing the resulting lysogen of TP21 by growth at a nonpermissive temperature. If we can apply this technique to TP21 lysogens of other B. anthracis strains that have a low sensitivity to the phage, we can test populations of cured cells for sensitivity to the phage. If the above hypothesis is correct, all progeny of cells cured in this manner should be sensitive to TP21.

TP21 mutants. Since TP21 formed such small and indistinct plaques on lawns of B. cereus 569, it seemed desirable to isolate a temperature inducible mutant which should form better plaques at the high temperature. The strategy used to isolate temperature-inducible mutants of TP21 was to assay at a high temperature a phage lysate obtained from a lysogenic culture mutagenized by NTG. This should allow temperature-inducible mutants to form plaques which appear clear when assayed at an elevated temperature.

By this procedure twenty clear-plaque variants were isolated and these were tested at a low temperature. Four isolates designated by the appendant notation c3, c7, c8 and c12 formed plaques which were less clear at 30°C then at 42°C. These were not true clear-plaque mutants since at a high multiplicity of infection (MOI) they could lysogenize B. cereus 569. At a similar MOI other clear-plaque mutants lysed B. cereus. These four TP21 plaque morphology mutants were tested for thermoinducibility. TP21c12 appeared to be somewhat temperature inducible. TP21c12 cultures grown at 42°C exhibited a 30-fold increase in phage titer when compared to a culture maintained at 30°C. At 42°C cultures of the other isolates either had a lower titer compared to the 30°C control or only a small increase.

These strains were easier to assay than wild-type TP21, which was the reason they were originally isolated. The discovery of the superior ability of B. cereus 569 UM20-1C1, cured of all plasmids, to serve as an indicator for wild-type TP21 made the temperature inducibility of these isolates less important. They were of value for other purposes described below.

Temperature sensitive replication mutant. TP21 lysogens appeared to be very stable. Cells cured of TP21 were never detected when colonies of lysogens were picked to plates spread with TP21c1 to test for immunity. Lysogenic strains stably maintained TP21 even following growth at 42°C in broth either with or without TP21 antiserum.

Scott (8) has found that lysogens of one class of conditional clear-plaque mutants of the coliphage P1 are cured when cells are grown at the nonpermissive temperature. With this in mind we tested lysogens of the TP21 conditional clear-plaque mutants TP21c3, TP21c7, TP21c8 and TP21c12 for curing during growth at high temperature. To screen these mutants for temperature sensitivity with respect to replication, cultures lysogenized with each of the respective phage mutants were grown from spores at 42°C in broth and then streaked for isolation onto NBY agar plates. Colonies were tested for the presence of TP21 prophage by picking 50 representative colonies to soft agar lawns of B. cereus 569. Lysogens carrying these mutants make clear zones when stabbed into lawns of B. cereus 569 and, as they are immune to TP21, will grow on plates spread with TP21c1. All 50 colonies of the TP21c3 lysogen made clear zones and therefore still carried the phage. Thirty three colonies of the TP21c8 lysogen made clear zones in the lawns and grew in the presence of TP21c1 on plates spread with the phage. This indicated that 34% of the cells

from the culture grown at 42°C were cured of the page. However, none of 50 colonies from the 42°C cultures of the lysogens carrying TP21c7 and TP21c12 was immune to TP21c1 and none produced zones of lysis in the indicator lawn, indicating a high percentage of curing among cells from the cultures lysogenized with these derivatives. These results indicated that the TP21 mutants TP21c7 and TP21c12 were temperature-sensitive for replication and suggested that they would be good candidates for use in creating a temperature-sensitive transposition-selection vector for Tn*917* mutagenesis.

Tagging the temperature-sensitive TP21 mutant with Tn917. The temperature-sensitive replication mutant TP21c7 was chosen for experiments to place Tn*917* on a curable replicon. This procedure was essentially identical to that in which Tn*917* was placed on wild-type TP21. The source of Tn*917* was pXO12::Tn*917*, carried by a *B. cereus* 569 strain which was lysogenized with TP21c7.

The isolation of TP21c7::Tn*917* was much more difficult than expected by comparison to the ease with which the wild-type TP21 was tagged with Tn*917*. Fifty MLS-resistant transductants carrying a tagged derivative of TP21c7 were isolated by transducing *B. cereus* 569 with "low frequency of transduction" (LFT) lysates obtained during the tagging procedure. To screen for viable transposon-tagged TP21c7, cell-free filtrates were harvested from the MLS-resistant transductants and tested for the ability to serve as "high frequency of transfer" (HFT) lysates. Four isolates produced HFT lysates; however, only one retained this ability after spore stocks were prepared. When examined by agarose gel electrophoresis, cells of the Tn*917*-tagged TP21 lysogens were found to carry newly acquired plasmid DNA which migrated at a rate consistent with that of other Tn*917*::TP21 derivatives.

Curing experiments were carried out with lysogens of TP21c7::Tn*917*. The results of these tests showed that the transposon-tagged mutant behaved like TP21c7 in that cells isolated following growth in broth at 42°C were no longer immune to TP21c1 indicating that they no longer contained prophage DNA. Cells isolated from cultures grown at 30°C were all immune to TP21c1, even when the growth medium contained TP21 antiserum. As expected MLS resistance and immunity were linked; all immune isolates were resistant to antibiotic, whereas all isolates losing immunity also lost MLS resistance.

Results presented earlier of experiments using a transposon-tagged derivative of wild-type TP21 (Annual Progress Report, July 1988) showed that

when selective pressure was applied the tagged derivative could replace the resident prophage of a TP21 lysogen. Analogous tests were performed using TP21c7::Tn917. TP21 lysogens of B. cereus 569 and B. anthracis 4229 as well as the natural host B. thuringiensis subsp. kurstaki HD1-9, were lysogenized with TP21c7::Tn917. Cells cured of TP21 phage were then isolated following growth of each lysogenic strain at 42°C.

For many reasons TP21c7::Tn917 seems as if it should serve as an ideal transposition selection vector. However, several preliminary experiments in which it was tested as a mutagenic vehicle have been unsuccessful. The reasons for this are not clear. It is conceivable that Tn917 in the temperature-sensitive mutant is defective in transposition. Further experiments will be carried out with TP21c7::Tn917 and with another temperature-sensitive tagged mutant of TP21 that has been isolated recently.

III. Further characterization of conjugative plasmid pLS20

We have continued to put a small effort into the study of pLS20, the fertility plasmid found in Bacillus subtilis (natto). The host range of this plasmid includes, in addition to B. subtilis, strains of B. anthracis, B. cereus, and B. thuringiensis. The fertility plasmid mediates transfer of plasmids among the various strains. Therefore, we are interested in using pLS20 in our studies of the mechanism of plasmid transfer among Bacillus species by conjugation. pLS20 is considerably smaller than the B. thuringiensis fertility plasmids and lends itself more readily to genetic manipulation.

Restriction endonuclease cleavage sites on pLS20. A survey of restriction endonuclease cleavage sites was done to determine the numbers of restriction fragments generated by various enyzmes. A total of 18 restriction endonucleases were used and the numbers of cleavage sites found for the enzymes are shown in Table 11. Results of the survey imply that the plasmid is A+T rich as enzymes such as BamHI and SmaI which recognize G+C-rich areas yielded few restriction fragments, whereas enzymes such as HindIII and EcoRI which recognize A+T-rich regions yielded more fragments.

Isolation of Tn917-tagged pLS20 derivatives. Following the induction of Tn917 transposition and curing of pTV1, cells were streaked to agar plates containing selective levels of erythromycin and then screened for their

sensitivity to chloramphenicol. The percentage of cells cured of pTV1 was 99.9%. Those cells that were MLS-resistant and chloramphenicol-sensitive were then subjected to analysis to determine whether Tn917 had transposed onto the plasmid or into the chromosome. Restriction endonuclease digestion of plasmid DNA isolated from these transposants confirmed that the insertion of Tn917 onto plasmid rather than chromosomal DNA occurred 60% of the time. Among eighty independently generated pLS20::Tn917 derivatives isolated in these experiments thirteen unique insertions were found.

Isolation of transfer-positive Tn917-tagged derivatives. Although there were thirteen unique, independently isolated Tn917-tagged pLS20 plasmid derivatives available for study, it was of interest to obtain more MLS-resistant plasmid derivatives. Selection of transposants as described above by a procedure that did not rely on the retention of conjugative functions yielded predominantly insertions that were clustered in one region of the plasmid. This region, the 10.8-kb BglII fragment, has been directly implicated as being necessary for fertility. Insertions of the transposon into this region affected the ability of the plasmid to mediate plasmid DNA transfer. The transposon preferentially inserts within this region, thus identifying a "hot region" for transposition of Tn917. In contrast, when insertions of Tn917 were selected on the basis of transfer of MLS resistance, i.e., insertions that left fertility functions intact, fewer transposants were found.

To obtain insertions into other regions of pLS20 that do not affect conjugal transfer ability, we exposed B. subtilis cells harboring pLS20 and pTV1 to the usual transposition and curing procedure. The cells were then used directly as inoculum for a donor culture. The mating recipients were B. subtilis strains IG-20 UM1 and 168 UM21. Each mating was plated on eight membranes, with selection for the recipient markers and the acquisition of MLS resistance. Two MLS^r IG-20 UM1 transcipients and 4 MLS^r 168 UM21 transcipients were obtained. These transcipients were streaked to selective plates for isolation and purification. Agarose gel electrophoresis of plasmid DNA digested with BglII identified 4 new insertions of Tn917 within pLS20. Three of these insertions were within the largest BglII fragment and one was within the 5.4-kb BglII fragment. These plasmids were denoted pXO514 - pXO517. The 4 MLS-resistant transcipients were then used as donors in membrane matings with B. subtilis recipients to confirm the Tra^+ phenotype.

In all cases, the transfer frequency of MLS resistance was comparable to that observed when the donor plasmid was pXO503 or pXO504 whose fertility is comparable to that of the wild-type plasmid.

Sites of Tn917 insertions in pLS20. The sites of Tn917 insertion in pLS20 were first determined by restriction of pLS20::Tn917 derivatives with the restriction endonucleases BglII and AvaI. These enzymes were chosen because of the location of their recognition sites within the transposon. Tn917 contains 3 (or 4 depending on the host organism) cleavage sites for AvaI. The rationale for choosing this enzyme was that 2 of the cleavage sites for AvaI are located 7 base pairs from each end of the transposon, thus essentially "popping out" the transposon from the fragment into which it has inserted. BglII was chosen because of the size of the Tn917 fragments it generates. This enzyme cuts the transposon at two sites, yielding a 1.7-kb fragment representing the internal portion of the transposon, and leaving 2.8 and 0.7 kb of Tn917 DNA on two pLS20 DNA fragments. This enzyme provided an easy way to screen for the presence of the transposon in the plasmid by looking for the characteristic 1.7-kb internal fragment as well as providing a way to later orient the transposon in the tagged plasmid derivatives.

Fertility of strains containing pLS20::Tn917 derivatives. B. subtilis cells harboring the transposon-tagged pLS20 plasmid derivatives pXO501 - pXO511 were employed as donors in intraspecific matings to determine whether the insertion of Tn917 had interrupted any DNA sequences necessary for transfer. Selection for transfer of the fertility plasmid (MLS resistance transfer) or pBC16 (tetracycline resistance transfer) was applied. Table 12 shows the results of these mating experiments.

The different transfer phenotypes exhibited by the various pLS20::Tn917-containing B. subtilis hosts could be due to either i) insertional inactivation of a required transfer function by Tn917 or ii) varying degrees of conjugative plasmid stability within the B. subtilis donor cells. To differentiate between these possibilities, donor cell counts were determined on nonselective medium and on medium containing the appropriate antibiotics. The results indicated that most of the transposon-tagged plasmids were stably maintained in their hosts. Of the plasmids examined, pXO503 was the most unstable, yet this plasmid conferred one of the highest levels of fertility upon its host. Therefore, the reduction in or loss of fertility exhibited by donor cells harboring these tagged derivatives was due not to the loss of

these plasmids from the host cells but to the insertional inactivation of pLS20 DNA sequences required for conjugal transfer proficiency.

Classes of pLS20::Tn917 derivatives. As shown in Table 12 the various transposon-tagged pLS20 derivatives could be divided into three classes based upon their transfer proficiencies. Class I plasmids contain an insertion of Tn917 within the 10.8-kb BglII fragment which eliminated almost all pLS20-mediated plasmid transfer. Possibly the transposon has interrupted a trans-acting factor, such as a site-specific endonuclease responsible for nicking plasmid DNA prior to transfer. Examples of this plasmid class include pXO505, pXO507, pXO509, and pXO510. Class II plasmids contain an insertion of Tn917 within the 10.8-kb BglII fragment which abolished or significantly reduced self-mobilization but did not significantly affect the mobilization of the non-conjugative plasmid pBC16. Possibly the transposon has interrupted a cis-acting factor, such as the oriT site, thus eliminating or reducing the mobilization of pLS20 and leaving the mobilization of other plasmids unaffected. Examples of this plasmid class are pXO501, pXO506, pXO508 and pXO511. Class III plasmids contain an insertion of Tn917 in a region outside of the 10.8-kb BglII fragment. Transfer frequencies of the conjugative plasmid and of pBC16 were comparable to those conferred upon the cell by wild-type pLS20. Examples of this plasmid class are pXO502, pXO503, and pXO504.

Formation of deletions. Passage of cells that harbored either pXO503 or pXO504 several times in LG broth supplemented with 1 μg of erythromycin per ml resulted in a deletion of 15-17 kilobases of pLS20 DNA. These two plasmids contain transposon insertions within the 27.0-kb BglII fragment proximal to the 10.8-kb BglII fragment involved in pLS20-mediated plasmid exchange. These deletions appear to initiate at the site of insertion of Tn917 and terminate near the distal end of the 10.8-kb BglII fragment.

All of the other transposon-tagged derivatives, most with an insertion inactivating some aspect of pLS20-mediated plasmid transfer, did not generate detectable deletion derivatives. The four recently derived transposon-tagged plasmids pXO514 - pXO517, which all exhibit normal transfer efficiencies, also do not appear to undergo detectable deletions as judged by agarose gel electrophoresis of plasmid lysates and BglII restriction digestions. There are two possible explanations for this: i) these plasmids are very stable and do not undergo deletion formation; or ii) these plasmids also undergo deletion

formation but the deletion involves DNA sequences required for plasmid maintanence and/or replication.

Requirement for recombination functions in recipients. Membrane matings were carried out utilizing B. subtilis strains harboring pBC16 and one of the Tn917-tagged Tra$^+$ pLS20 derivatives pXO501 through pXO504 as donors of MLS resistance to recipients that were proficient or deficient in recombination. Initially these matings were carried out to introduce pXO501 through pXO504 into the recombination-deficient strain B. subtilis PSL1 UM1 so that the effects of recombination functions on the formation of deletions could be investigated. However, none of these mating mixtures generated any MLS-resistant transcipients. Membrane matings were then carried out between B. subtilis donors harboring both pBC16 and one of the pLS20::Tn917 plasmids pXO501 through pXO504 and the recipients B. subtilis PSL1 UM1 Rec$^-$ and B. subtilis 168 Rec$^+$.

The donors harboring pXO501 through pXO504 were fertile. B. subtilis 168 Rec$^+$ cells were able to act as recipients for both the fertility plasmid and pBC16. However, PSL1 UM1 Rec$^-$ cells were not able to act as recipients for either the fertility plasmid or pBC16, indicating the necessity for recombination functions in the recipient cell for the mating event to occur or for the transferred plasmids to be established in the recipient.

Cloning experiments. Bacillus subtilis 168 UM1-1 was grown to a competent state and transformed with a ligation mixture containing pBD64 DNA linearized at its unique BglII site and the 10.8-kb BglII fragment of pLS20. The vector DNA is 4.8 kb and is a spontaneous deletion derivative of the 6.8-kb recombinant plasmid pBD12. Plasmid pBD64 is composed of most of the pUB110 genome and 1.0-kb of the pC194 genome encompassing the cat gene, thus conferring both kanamycin resistance and chloramphenicol resistance upon host organisms. This plasmid is useful as a cloning vehicle as it contains a single BglII site within the kanamycin resistance gene, allowing for insertional inactivation of this gene with BglII-digested DNA of interest while maintaining the selectable chloramphenicol resistance gene.

The recipient in the transformation contained pBC16. This plasmid shares homology with the pUB110 portion of the vector DNA. It has been reported that the presence of homologous DNA in the recipient allows for more efficient maintanence of transforming DNA due to homologous recombination events.

The transformation mixture was plated in soft agar containing chloramphenicol. The colonies which came up after 1 to 2 days of incubation were screened for their antibiotic resistances. Those that were found to be chloramphenicol resistant and kanamycin sensitive were tested for their ability to transfer chloramphenicol resistance in matings with B. subtilis 168 UM47 as the recipient. Two clones capable of transferring MLS resistance were obtained. These are currently being characterized further.

Motility of cells containing pLS20::Tn917 plasmids. All pLS20-containing strains of B. subtilis tested to date were non-motile. When cells containing the plasmid were planted in soft agar and allowed to grow to form a colony, occasionally motile cells would swarm from the edge of the colony. Plasmid extracts fron the motile cells showed that they were cured of pLS20. To determine whether Tn917 had interrupted the region of pLS20 responsible for suppression of motility, the pLS20::Tn917-containing B. subtilis cells were picked to the center of 0.4% agar plates and incubated for 2 days at 30xC.

As with parental plasmid pLS20, all the transposon-tagged plasmids suppressed the motility of the host organism. Apparently none of the insertions of Tn917 occurred within the region of DNA responsible for this suppression of motility. After several days, however, some motile outgrowth was observed from pXO503- and pXO504-containing cells. Examination of plasmid content of these cells indicated that the cells taken from the point of inoculation retained the parental pLS20::Tn917 plasmid. Cells from the outer edge of the plate were found to contain either a deleted form of the plasmid or to have been cured. Thus, the region of DNA responsible for the suppression of motility appears to be within the approximately 15 kb of pLS20 DNA that is lost in the deletion derivatives.

Can pLS20 promote mating events in liquid culture. To determine whether pLS20 would mediate plasmid transfer in broth matings, a series of interspecific and intraspecific matings with B. subtilis and B. anthracis were carried out. All similar combinations were known to be effective when matings were carried out on membranes. The results showed that pLS20 can promote the conjugal transmission of plasmid DNA in broth matings between a variety of B. subtilis strains; however, intraspecific broth matings between B. anthracis strains or interspecific broth matings between B. subtilis and B. anthracis strains was not observed. These experiments reveal that the B. thuringiensis mating system and the B. subtilis (natto) mating system are fundamentally

different, as the B. thuringiensis mating system is very effective in transferring plasmids in broth culture interspecifically as well as intraspecifically among strains of B. anthracis, B. cereus, and B. thuringiensis.

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain or plasmid	Relevant characteristics[a] and plasmids	Origin or Reference[b]
B. anthracis		
Weybridge	Avirulent, Tox^+ Cap^- pXO1	10
Weybridge UM44	Ind^- Tox^+ Cap^- pXO1	UV of Weybridge
Weybridge UM44-1	Ind^- Tox^+ Str^r, pXO1	UV of UM44
Weybridge UM44-2 (same as UM44-1C9)	Ind^- Tox^- Str^r, $(pXO1)^-$	Curing of UM44-1
Weybridge A	Colony variant of Weybridge, Tox^+, pXO1	10
Weybridge A UM14	Phe^- Tox^+, pXO1.1	UV of Weybridge A
Weybridge A UM14C1	Phe^- Tox^-, $(pXO1.1)^-$	C. B. Thorne
Weybridge A UM18	pXO1.1, $pXO2^-$, Car^-	C. B. Thorne
Weybridge A UM23	Ura^- Tox^+, pXO1.1	UV of Weybridge A
Weybridge A UM23-1	Ura^- Tox^+, pXO1.1 Str^r	UV of UM23
Weybridge A UM23-2 (same as A UM23C1)	Ura^- Tox^-, $(pXO1.1)^-$	Curing of A UM23
Weybridge A UM23-3 (same as A UM23C1-1)	Ura^- Tox^- Str^r, $(pXO1.1)^-$	UV of A UM23-2
4229 (Pasteur)	Cap^+ Tox^-, pXO2	4

Continued next page

Table 1 (continued)

4229R1	$pXO2^-$, $pXO1^-$	Spontaneous
Ames ANR-1	pXO1, $pXO2^-$	USAMRIID
ΔAmes-1	pXO2, $pXO1^-$	USAMRIID
New Hampshire NNR-1	pXO1, $pXO2^-$	USAMRIID
New Hampshire ΔNH-1	pXO2, $pXO1^-$	USAMRIID
New Hampshire NNR-1Δ-1	$pXO1^-$, $pXO2^-$	USAMRIID
M UM2	pXO2, $pXO1^-$	T. Koehler
M UM6	pXO1, $pXO2^-$	T. Koehler
Vollum PM36R-1	pXO1, $pXO2^-$	C. Thorne
6602 (Pasteur)	Cap^+ Tox^-, pXO2	4
B. cereus		
569	Wild type	NRRL
569 K	Trp^- Str^r	A. Aronson
569 UM20-1	Ant^- Str^r	C. B. Thorne
569 UM20-1C1	Ant^- Str^r, Cured of all plasmids	This study
569 L21(TP21)	TP21 lysogen	This study
T	wild-type	I. Goldberg
4415	wild-type	ATCC
13472	wild-type	ATCC

Continued next page

Table 1 (continued)

B. subtilis		
PSL1	r^- m^- Arg$^-$ Thr$^-$ Leu$^-$ Rec$^-$	BGSC strain 1A510
PSL1 UM1	r^- m^- Arg$^-$ Thr$^-$ Leu$^-$ Str^r Rec$^-$	Spontaneous from PSL1
PSL1 UM3	PSL1(pLS20, pTV1, pBC16)	T. Koehler
IG-20	r^- m^- Trp$^-$	BGSC strain 1A436
IG-20 UM1	r^- m^- Trp$^-$ Rif^r	UV of IG-20
168	trpC2	M. Fox
168 UM1	trpC2 Str^r	Spontaneous from 168
168 UM1-1	trpC2 Str^r Tc^r pBC16	Transformation of 168 UM1
168 UM21	Leu$^-$ Met$^-$ Str^r	C. B. Thorne
168 UM47	trpC2 Rif^r	T. Koehler
Bacillus subtilis (natto)		
3335	Bio$^-$	T. Hara
B. thuringiensis		
subsp. kurstaki HD1-9	Carries phage TP21	A. Aronson
subsp. finitimus 4040	wild-type	NRRL
subsp. alesti 4041	wild-type	NRRL
subsp. thuringiensis 4042A	wild-type	NRRL

Continued next page

Table 1 (continued)

subsp. aizawai 4042B	wild-type	NRRL
subsp. sotto 4042C	wild-type	NRRL
subsp. dendrolimus 4043	wild-type	NRRL
subsp. kenyae 4044	wild-type	NRRL
subsp. galleriae 4045	wild-type	NRRL
subsp. entomocidus 4046	wild-type	NRRL
subsp. entomocidus-limassol 4047	wild-type	NRRL
subsp. aizawai 4048	wild-type	NRRL
subsp. morrisoni 4049	wild-type	NRRL
subsp. tolworthi 4050	wild-type	NRRL
subsp. canadensis 4056	wild-type	NRRL
subsp. subtoxicus 4057	wild-type	NRRL
subsp. darmstadiensis 4058	wild-type	NRRL
subsp. toumanoffi 4059	wild-type	NRRL
subsp. thompsoni 4060	wild-type	NRRL
subsp. israelensis BIS	wild-type	M. deBarjac

Continued next page

Table 1 (continued)

Bacteriophages		
CP-51	Generalized transducing phage	C. B. Thorne
CP-51*ts*45	Temperature-sensitive mutant of CP-51	C. B. Thorne
TP-21	Prophage is a plasmid	*B. thuringiensis kurstaki* HD1-9
TP21c1	Clear-plaque mutant of TP21	This study
TP21c3	Plaque-morphology mutant	This study
TP21c7	Plaque-morphology mutant, temperature sensitive	This study
TP21c8	plaque-morphology mutant	This study
TP21c12	plaque-morphology mutant, temperature sensitive	This study
TP21-20	TP21::Tn*917*	This study
Plasmids		
pBC16	Encodes tetracycline resistance	2
pBD64	Cloning vector, hybrid of pUB110 and pC194	P. Lovett
pLS20	Conjugative plasmid of *B. subtilis* (*natto*)	6
pTV1	Temperature-sensitive transposition selection vector	Youngman
pXO1	Toxin plasmid from *B. anthracis* Weybridge	7, 10
pXO1.1	Toxin plasmid from Weybridge A mutants	This study

Continued next page

Table 1 (continued)

pXO2	Encodes synthesis of B. anthracis capsule	4
pXO12	Tra^+ Cry^+ from B. thuringiensis 4042A	1
pXO501 to pXO503	pLS20::Tn917	6
pXO504 to pXO517	pLS20::Tn917	This study

[a] Abbreviations: Ade, adenine; Ant, anthranilic acid; Ind, indole; Trp, tryptophan; Ura, uracil; MLS^r, Tn917-encoded macrolide, lincosamide, and streptogramin B resistance; Rif^r, rifampicin resistant, Str^r, streptomycin resistant; Tc^r, pBC16 encoded tetracycline resistance; Cap, synthesis of polyglutamate capsule; Tox, synthesis of anthrax toxin..

[b] BGSC, Bacillus Genetics Stock Center, Columbus, Ohio; NRRL, Northern Regional Research Laboratory, Department of Agriculture, Peoria, IL; USAMRIID, US Army Medical Research Institute of Infectious Diseases; UV, mutagenesis by UV light (9).

TABLE 2. Auxotrophic mutants resulting from Tn*917* chromosomal insertions

Strain[a]	Relevant plasmids	Relevant characteristics[b]
B. anthracis Weybridge A		
UM23C1 td20[c]	pTV1	Ura$^-$, Cm^r, MLS^r
UM23C1 td20C1	(pTV1)$^-$	Ura$^-$, *pur*::Tn*917*, Cm^s, MLS^r
UM23C1 td20C2	(pTV1)$^-$	Ura$^-$, *rib*::Tn*917*, Cm^s, MLS^r
UM23C1 td20C3	(pTV1)$^-$	Ura$^-$, *rib*::Tn*917*, Cm^s, MLS^r
UM23C1 td25[c]	pTV1*ts*	Ura$^-$, Cm^r, MLS^r
UM23C1 td25C1	(pTV1*ts*)$^-$	Ura$^-$, *aro*::Tn*917*, Cm^s, MLS^r
UM23C1 td25C2	(pTV1*ts*)$^-$	Ura$^-$, Pig$^+$, *spo*::Tn*917*, Cm^s, MLS^r
UM23C1 td25[c]	pTV1*ts*	Ura$^-$, Spo$^-$, Cm^r, MLS^r
UM23C1 td25C3	(pTV1*ts*)$^-$	Ura$^-$, Spo$^-$, *pur*::Tn*917*, Cm^s, MLS^r
UM23C1 td25C4	(pTV1*ts*)$^-$	Ura$^-$, Spo$^-$, *gua*::Tn*917*, Cm^s, MLS^r
UM23C1 td25C5	(pTV1*ts*)$^-$	Ura$^-$, Spo$^-$, *rib*::Tn*917*, Cm^s, MLS^r
UM23C1 td25C6	(pTV1*ts*)$^-$	Ura$^-$, Spo$^-$, *rib*::Tn*917*, Cm^s, MLS^r
B. cereus 569		
UM20-1C1 td6[c]	pTV1*ts*	Ant$^-$, Str^r, Cm^r, MLS^r
UM20-1C1 td6C1	(pTV1*ts*)$^-$	Ant$^-$, *rib*::Tn*917*, Str^r, Cm^s, MLS^r
UM20-1C1 td6C2	(pTV1*ts*)$^-$	Ant$^-$, *pur*::Tn*917*, Str^r, Cm^s, MLS^r
UM20-1C1 td6C3	(pTV1*ts*)$^-$	Ant$^-$, *leu*::Tn*917*, Str^r, Cm^s, MLS^r

[a] In the strain designations td denotes transductants obtained by CP-51 mediated transfer of pTV1.

[b] Abbreviations: Cm^r, chloramphenicol resistance. MLS^r, Tn*917* encoded macrolide, lincosamide and streptogramin B resistance. Str^r, streptomycin resistance. Ura, uracil; Pur, purine; Rib, riboflavin; Aro, aromatic; Gua, guanine; Pig, pigment; Ant, anthranilic acid; Leu, leucine; Spo, spore formation.

[c] Parent strains used to generate insertions.

TABLE 3. Estimated sizes of PstI and BamHI restriction fragments of pXO1.1 from B. anthracis Weybridge A UM23

PstI		BamHI	
Fragment	Size (kb)	Fragment	Size (kb)
1	18.7	1	38.7
2	18.1	2	34.8
3	16.2	3	26.2
4	11.6	4	19.9
5,6	10.7	5	14.6
7	10.1	6	13.9
8,9	9.3	7	7.7
10,11	6.6	8	7.4
12	6.3	9	7.1
13	6.0	10	6.6
14	5.7	11	6.0
15	5.5	12	3.9
16	5.2		
17	4.7	Total	186.8 kb
18	4.1		
19	3.4		
20	2.8		
21	2.1		
22	1.7		
23	1.3		
24	1.1		
25	1.0		
26	0.8		
27	0.7		
28	0.5		
29	0.4		
30	0.3		
Total	181.5 kb		

TABLE 4. Phenotypic characteristics of Weybridge A strains carrying pXO1.1::Tn*917*

Strain[a]	Phenotypic characteristics			
	Halo assay[b]	PA production[c]	Sporulation[d] at 37°C	Sensitivity to CP-51*ts*45[e]
A UM23(pXO1.1)	+	1:8	Osp	-
A UM23C1(pXO1.1)$^-$	-	0	Spo$^+$	+
A UM23 tp1A	+	1:8	Osp	-
A UM23 tp2A	+	1:8	Osp	-
A UM23 tp2	+	1:8	Osp	-
A UM23 tp5	+	1:8	Osp	-
A UM23 tp6	+	1:4	Osp	-
A UM23 tp8	+	1:8	Osp	-
A UM23 tp10	+	1:8	Osp	-
A UM23 tp12	+	1:8	Osp	-
A UM23 tp14	+	1:8	Osp	-
A UM23 tp15	+	1:8	Osp	-
A UM23 tp16	+	1:8	Osp	-
A UM23 tp17	+	1:8	Osp	-
A UM23 tp18	-	0	Osp	-
A UM23 tp20	+	1:8	Osp	-
A UM23 tp21	-	0	Osp	-
A UM23 tp22	+	1:8	Osp	-
A UM23 tp25	+	1:8	Osp	-
A UM23 tp26	-	0	Osp	-
A UM23 tp27	+	1:4	Osp	-[f]
A UM23 tp28	+	1:8	Osp	+[f]
A UM23 tp29	-	0	Osp	-
A UM23 tp30	+	1:8	Osp	-
A UM23 tp32	-	0	Osp	-
A UM23 tp36	+	1:8	Osp	-
A UM23 tp37	+	1:8	Osp	-
A UM23 tp38	-	0	Osp	-
A UM23 tp39	-	0	Osp	-
A UM23 tp40	+	1:8	Osp	-
A UM23 tp41	+	1:8	Osp	-

[a] tp, transposant.

[b] +, strains produced protective antigen (PA$^+$); -, PA$^-$ strains.

[c] PA, protective antigen; 0, no PA detected; 1:4 or 1:8 denotes the highest dilution of culture filtrate which produced a visible precipitin line in the Ouchterlony double diffusion assay.

[d] Osp, oligosporogenous; Spo$^+$,extensive sporulation.

[e] +, plaque formation; -, no detectable plaques.

[f] Plaques were very turbid compared to plaques on a pXO1.1$^-$ strain.

TABLE 5. PstI and BamHI restriction fragments of pXO1.1::Tn917 derivatives from Weybridge A UM23 transposants

Strain	PstI fragments		BamHI fragments		Altered phenotype
	Altered	New	Altered	New	
UM23 tp1A	10.1	15.0[a]	38.7	46.3[a]	no
UM23 tp2A	5.2	10.1[a]	34.8	39.8[a]	no
UM23 tp15	9.3, 4.7, 2.1	14.0	19.9	17.6[a]	no
UM23 tp21	18.1, 6.0	14.0[a]	34.8, 13.9, 6.0	44.2[a]	PA^{-b}, EF^{+b}, LF^{+b}
UM23 tp27	18.1	23.3	13.9	19.5[a]	no
UM23 tp28	9.3	14.6[a]	6.6	12.1[a]	CP-51 sensitive
UM23 tp29	18.1	23.3[a]	13.9	19.5[a]	$PA^{+/-b}$, EF^{-b}, $LF^{+/-b}$
UM23 tp32	18.1	23.3	13.9	19.5[a]	PA^{-b}, EF^{+b}, $LF^{+/-b}$
UM23 tp36	6.3	11.6	34.8	39.8[a]	no
UM23 tp39	18.1, 6.0, 5.5	7.2	34.8, 13.9, 7.4 6.0	39.8	PA^{-b}, EF^{-b}, LF^{-b}

[a] These new fragments have been shown by DNA-DNA hybridizations to contain Tn917. The remaining new fragments have not yet been checked by hybridization.

[b] Determined by Dr. S. Leppla from USAMRIID

TABLE 6. Phenotypic characteristics of Weybridge strains carrying deletion derivatives of pXO1.1::Tn*917*

Strain[a]	Origin of tagged plasmid[b]	Phenotypic Characteristics			
		Halo assay[c]	PA production[d]	Sporulation[e] at 37°C	Sensitivity to CP-51*ts*45
A UM23(pXO1.1)		+	1:8	Osp	-
A UM23C1(pXO1.1)$^-$		-	0	Spo$^+$	+
A UM14(pXO1.1)		+	1:16	Osp	-
A UM14C1(pXO1.1)$^-$		-	0	Spo$^+$	+[g]
UM44-1(pXO1)		ND[h]	1:16	Spo$^+$	+
UM44-1C9(pXO1)$^-$		ND[h]	0	Spo$^+$	+
A UM23C1 tds1	A UM23 tp2A	-	0	Osp	-
A UM23C1 tds2	A UM23 tp2A	-	0	Spo$^+$	+[g]
A UM23C1 tds4a	A UM23 tp1A	-	0	Spo$^+$	+
A UM23C1 tds4b	A UM23 tp1A	-	0	Spo$^+$	+
A UM23C1 tds4c	A UM23 tp1A	-	0	Spo$^+$	+
A UM23C1 tds4d	A UM23 tp1A	-	0	Spo$^+$	+
A UM23C1 tds5	A UM23 tp21	-	ND[h]	Osp	-
A UM23C1 tds6	A UM23 tp2A	-	0	Spo$^+$	+[g]
A UM23C1 tds9	A UM23 tp1A	-	0	Spo$^+$	+
A UM14C1 tds1	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds2	A UM23 tp1A	-	0	Osp	-

(Continued on next page)

Table 6 (continued)

A UM14C1 tds3	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds4	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds5	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds6	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds7	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds8	A UM23 tp1A	-	0	Osp	-
A UM14C1 tds9	A UM23 tp2A	-	0	Osp	-
A UM14C1 tds10	A UM23 tp15	-	0	Osp	-
A UM14C1 tds11	A UM23 tp15	-	0	Osp	-
A UM14C1 tds12	A UM23 tp15	-	0	Osp	-
A UM14C1 tds13	A UM23 tp15	-	0	Osp	-
A UM14C1 tds14	A UM23 tp15	-	0	Spo^+	$+^g$
A UM14C1 tds15	A UM23 tp15	-	0	Osp	-
A UM14C1 tds16	A UM23 tp15	-	0	Osp	-
A UM14C1 tds17	A UM23 tp15	-	0	Osp	-
UM44-1C9 tds2	A UM23 tp1A	-	0	Spo^+	-
UM44-1C9 tds3	A UM23 tp1A	-	0	Spo^+	-
UM44-1C9 tds4	A UM23 tp1A	-	0	Osp	-
UM44-1C9 tds5	A UM23 tp1A	-	0	Osp	-
UM44-1C9 tds6	A UM23 tp1A	-	0	Osp	-
UM44-1C9 tds7	A UM23 tp1A	-	0	Osp	-
UM44-1C9 tds8	A UM23 tp15	-	0	Osp	-

(Continued on next page)

TABLE 6 (continued)

UM44-1C9 tds9	A UM23 tp15	-	0	Osp	-
UM44-1C9 tds10	A UM23 tp15	-	0	Osp	-
UM44-1C9 tds11	A UM23 tp15	-	0	Osp	-
UM44-1C9 tds12	A UM23 tp21	-	0	Osp	-

[a] tds, strains containing transductionally shortened pXO1.1::Tn917.

[b] See Table 4 of this report.

[c] +, strains produced protective antigen (PA^+); -, PA^- strains.

[d] PA, Protective Antigen. 0, no PA detected. 1:8 or 1:16 denotes the highest dilution of culture filtrate which produced a visible precipitin line in the Ouchterlony double diffusion assay.

[e] Osp, oligosporogenous; Spo^+, extensive sporulation.

[f] +, plaque formation; -, no detectable plaques.

[g] Plaques were more turbid than those observed on A UM23C1.

[h] ND, Not determined.

TABLE 7. Estimated sizes of pXO1::Tn*917* deletion derivatives as determined by *Pst*I and *Bam*HI digestions

Source of plasmid	Sum of fragment sizes (kb) generated by *Pst*I	Sum of fragment sizes (kb) generated by *Bam*HI
A UM23(pXO1.1)	181.5	186.8
pXO1.1::Tn*917*	186.7[a]	192.0[a]
A UM23C1 tds1	81.1	83.6
A UM23C1 tds2	127.3	130.8
A UM23C1 tds4d	140.9	146.4
A UM23C1 tds5	171.4	176.3
A UM23C1 tds6	116.8	119.7
A UM23C1 tds9	139.0	145.5

[a] The hypothetical size of pXO1.1 containing Tn*917* (5.2 kb)

TABLE 8. PstI restriction analysis of pXO1.1::Tn917 deletion derivatives

PstI fragments from pXO1.1[a]	pXO1.1 fragments (kb) not found in deleted plasmid from					
	tds1[b]	tds2[b]	tds6[b]	tds4d[c]	tds9[c]	tds5[d]
18.7	18.7	18.7	18.7		18.7	
18.1	18.1	18.1	18.1	18.1	18.1	18.1
16.2						
11.6						
10.7	10.7					
10.7						
10.1				10.1[e]	10.1[e]	
9.3	9.3	9.3	9.3	9.3	9.3	
9.3	9.3					
6.6	6.6	6.6	6.6	6.6	6.6	
6.6	6.6					
6.3						
6.0	6.0	6.0	6.0	6.0	6.0	6.0
5.7						
5.5	5.5	5.5	5.5	5.5	5.5	
5.2	5.2[e]	5.2[e]	5.2[e]			
4.7	4.7					
4.1						
3.4						
2.8						
2.1	2.1					
1.7						
1.3	1.3		1.3			
1.1						
1.0	1.0					
0.8						
0.7	0.7					
0.5						
0.4	0.4					
0.3						
	New PstI fragments (kb)					
	5.8[f]	15.2[f]	6.0[f]	15.0[f,g]	16.8 15.0[f,g]	14.0[f,g]

[a] PstI fragments generated from pXO1.1 of Weybridge A UM23.
[b] Origin of tagged plasmid was A UM23 tp2A.
[c] Origin of tagged plasmid was A UM23 tp1A.
[d] Origin of tagged plasmid was A UM23 tp21.
[e] Altered fragment in Tn917-tagged pXO1.1 from the donor transposant.
[f] Location of Tn917 determined by DNA-DNA hybridization.
[g] Location of Tn917 in tagged plasmid from the donor transposant.

TABLE 9. BamHI restriction analysis of pXO1.1::Tn917 deletion derivatives

BamHI fragments from pXO1.1[a]	pXO1.1 fragments (kb) not found in deleted plasmid from					
	tds1[b]	tds2[b]	tds6[b]	tds4d[c]	tds9[c]	tds5[d]
38.7				38.7[e]	38.7[e]	
34.8	34.8[e]	34.8[e]	34.8[e]	34.8	34.8	34.8
26.2						
19.9	19.9					
14.6	14.6	14.6	14.6	14.6	14.6	
13.9	13.9	13.9	13.9	13.9	13.9	13.9
7.7	7.7			7.7		
7.4	7.4	7.4	7.4	7.4	7.4	
7.1	7.1					
6.6	6.6					
6.0	6.0	6.0	6.0	6.0	6.0	6.0
3.9	3.9					
	New PstI Fragments (kb)					
	18.7[f,h]	20.7[f,h]	17.3[f,h]	46.3[f,g] 28.7[h]	46.3[f,g] 27.8[h]	44.2[f,g,h]

[a] BamHI fragments generated from pXO1.1 of Weybridge A UM23.
[b] Origin of tagged plasmid was A UM23 tp2A.
[c] Origin of tagged plasmid was A UM23 tp1A.
[d] Origin of tagged plasmid was A UM23 tp21.
[e] Altered fragment in Tn917-tagged pXO1.1 from the donor transposant.
[f] Location of Tn917 determined by DNA-DNA hybridization.
[g] Location of Tn917 in tagged plasmid from the donor transposant.
[h] Homologous to the 34.8-kb BamHI fragment as determined by DNA-DNA hybriziation.

TABLE 10. Sensitivity of various strains to TP21

Strain	TP21c1 Spot test reaction[a]	Frequency of MLS^r transduction by TP21::Tn917[b]
B. anthracis		
Vollum PM36 R1(pXO1))pXO2)$^-$	+/-	1×10^{-3}
Ames ANR-1(pXO1)(pXO2)$^-$	-	1×10^{-4}
ΔAmes-1(pXO2)(pXO1)$^-$	ND[c]	0
M UM6(pXO1)(pXO2)$^-$	-	8×10^{-4}
M UM2(pXO2)(pXO1)$^-$	ND	4×10^{-5}
New Hampshire NNR-1(pXO1)(pXO2)$^-$	ND	4×10^{-4}
New Hampshire ΔNH-1(pXO2)(pXO1)$^-$	ND	3×10^{-4}
New Hampshire NNR-1Δ1(pXO1, pXO2)$^-$	+/-	4×10^{-4}
Weybridge M18(pXO1)	-	3×10^{-4}
Weybridge M44-1(pXO1)	-	4×10^{-4}
4229(pXO2)	+	ND
4229R1(pXO2)$^-$	+	1×10^{-1}
B. cereus		
569	+	1
569 L21(TP21)	-	1×10^{-2}
569K	+	6×10^{-1}
4415	+	ND
T	-	0
13472	+	ND
B. thuringiensis		
subsp. *kurstaki* HD1-9(TP21)	ND	3×10^{-2}
subsp. *finitimus* 4040	+	R[d]
subsp. *alesti* 4041	+	R
subsp. *thuringiensis* 4042A	-	8×10^{-5}
subsp. *aizawai* 4042B	+	1×10^{-2}
subsp. *entomocidus* 4046	-	0
subsp. *entomocidus-limassol* 4047	+	R
subsp. *sotto* 4042C	-	ND
subsp. *dendrolimus* 4043	+	2×10^{-4}

Continued next page

Table 10 (Continued)

subsp. *kenyae* 4044	+	1×10^{-1}
subsp. *galleriae* 4045	+	R
subsp. *aizawai* 4048	+	0
subsp. *morrisoni* 4049	ND	1×10^{-2}
subsp. *tolworthi* 4050	ND	2×10^{-3}
subsp. *canadensis* 4056	-	0
subsp. *subtoxicus* 4057	+	9×10^{-2}
subsp. *darmstadiensis* 4058	+	R
subsp. *toumanoffi* 4059	ND	3×10^{-1}
subsp. *thompsoni* 4060	-	0
subsp. *israelensis*	ND	0

[a] A drop of lysate containing the clear-plaque mutant TP21c1 was spotted onto a lawn of either cells or spores for each strain. Turbidity less than that in the surrounding lawn indicated a positive reaction. A strong reaction is designated +; a weak reaction is designated +/-; no reaction is designated -.

[b] The frequency was determined as the number of MLS-resistant transductants per phage. The number of *B. cereus* 569 MLS^r transductants was taken as the number of specialized transducing phage.

[c] Not determined.

[d] Resistant to erythromycin and thus unable to be tested.

TABLE 11. Restriction endonuclease cleavage sites on pLS20.

Enzyme	No. of fragments[a] generated	Enzyme	No. of fragments generated
AvaI	5	PvuII	>20
BamHI	4	PstI	11
BclI	>15	SalI	1
BglII	5	SmaI	2
ClaI	>15	StuI	3
EcoRI	27	TaqI	1
HindIII	21	XhoI	3
KpnI	9	XbaI	2

[a] The number of restriction fragments was quantitated by running samples of the digestion mixture on electrophoretic gels containing 0.3%, 0.6% and 1.2% agarose to insure that all fragments were detected.

TABLE 12. Transfer abilities of B. subtilis donors harboring pLS20 derivatives[a]

Donor plasmid	MLS^r transcipients[a]		Tc^r transcipients[a]	
	per ml	Tc^r/MLS^r	per ml	MLS^r/Tc^r
Class I				
pXO505, pBC16	2.0×10^1	0/2	5.0×10^1	0/5
pXO507, pBC16	3.0×10^1	0/3	2.0×10^1	0/2
pXO509, pBC16	2.0×10^1	1/2	1.0×10^1	1/1
pXO510, pBC16	0	NA[b]	0	NA
Class II				
pXO501, pBC16	6.0×10^1	3/6	2.4×10^2	2/15
pXO506, pBC16	0	NA	1.4×10^2	0/18
pXO508, pBC16	0	NA	6.1×10^2	0/34
pXO511, pBC16[c]	3.1×10^2	17/21	6.9×10^3	0/22
Class III				
pXO502, pBC16	3.3×10^3	14/25	8.0×10^3	17/25
pXO503, pBC16	3.0×10^3	18/30	2.0×10^4	15/20
pXO504, pBC16	5.6×10^3	14/30	2.0×10^3	25/30
pXO514	6.0×10^2	NA	NA	NA
pXO515	3.2×10^4	NA	NA	NA

Continued on next page

Table 12 (Continued)

pX0516	1.0×10^3	NA	NA	NA
pX0517	2.4×10^4	NA	NA	NA
pLS20, pBC16	NA	NA	8.0×10^3	12/20

[a] The numbers of transcipients are the average obtained from at least 3 membrane mating experiments.

[b] NA, not applicable.

[c] pX0511 was included in this class because unless direct selection was applied, the tagged fertility plasmid was not observed to transfer. Although this does not correspond to abolishment of self-transfer, it implies an aberrant self-transfer and/or maintenance deficiency.

PUBLICATIONS

The following paper and abstracts were published during this reporting period:

1. Heemskerk, D. D., and C. B. Thorne. 1989. Genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Abstr. Annu. Meet. Am. Soc. Microbiol. H-269.

2. Ruhfel, R. E., and C. B. Thorne. 1989. Isolation of a temperature-sensitive replication mutant of phage TP-21 and a Tn917-tagged derivative. Abstr. Annu. Meet. Am. Soc. Microbiol. H-211.

3. Hornung, J. M., A. I. Guaracao-Ayala, and C. B. Thorne. 1989. Transposon mutagenesis in Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. H-256.

4. Green, B. D., L. Battisti, and C. B. Thorne. 1989. Involvement of Tn4430 in the transfer of Bacillus anthracis plasmids mediated by the Bacillus thuringiensis plasmid pXO12. J. Bacteriol. 171:104-113.

The following Ph. D. dissertation was written on research carried out under this contract:

Ruhfel, Robert E. Physical and genetic characterization of the Bacillus thuringiensis subsp. kurstaki HD-1 extrachromosomal temperate phage TP21. Ph. D. Dissertation. University of Massachusetts, Amherst. September 1989.

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